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Genome-linked protein VPg of poliovirus is present as free VPg and VPg-pUpU in poliovirus-infected cells

(RNA replication/picornavirus)

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ABSTRACT **VPg is a virus-encoded protein covalently attached to the 5′ end of poliovirus virion RNA. We have used antibody prepared against chemically synthesized VPg to detect two forms of VPg in infected cells. Both forms were specifically immunoprecipitated from lysates of infected cells labeled with [^{3}H]-leucine. One appears to be unmodified VPg because it had the same electrophoretic mobility as synthetic VPg. The other had a larger apparent molecular weight than VPg and could be labeled *in vivo* with $^{32}P_i$. Its structure is VPg-pUpU, the UMP dinucleotide being attached to VPg via a phosphodiester bond to tyrosine, the third amino acid from the NH_2 terminus of VPg. This structure is identical to that found at the 5′ end of virion and minus-strand RNA.**

A 22-amino acid protein, VPg, is attached to the 5′ end of the single-stranded RNA genome of poliovirus (1–3). VPg is linked to viral RNA by a tyrosine-phosphate bond in the structure, VPg(Tyr-O^4)-pU-U-A-A . . . (4, 5). VPg is also found at the 5′ end of intracellular plus and minus strand RNA, and on nascent plus strands; these observations suggest that VPg may act as a primer of poliovirus RNA synthesis (2, 3, 6).

The intracellular forms of VPg and their possible role in viral replication have been studied previously using antibodies made to chemically synthesized segments of VPg (7–9). These antibodies detected no free VPg in infected cells but specifically immunoprecipitated three larger polypeptides with VPg determinants from infected cell lysates, of which the smallest one had a molecular weight of 12,000 (pre-VPg 3 or P3-9) (7–9). In poliovirus replicase reactions, anti-VPg antibody specifically inhibited initiation of viral RNA synthesis and immunoprecipitated polynucleotides synthesized by the viral replicase (10). These results imply that protein with VPg determinants is attached to replicase reaction products and may initiate RNA synthesis. The inability to detect free VPg in infected cells suggested that one or more of the larger VPg-containing polypeptides initiates RNA synthesis (7, 11). We report here that free VPg as well as a discrete molecule with the structure VPg-pUpU is present in poliovirus-infected cells.

MATERIAL AND METHODS

Poliovirus was grown in suspension cultures of HeLa cells. Cells were labeled with $^{32}P_i$ (500 μCi/ml; 1 Ci = 37 GBq) 1–5 hr after infection or with [^{3}H]leucine (350 μCi/ml) 3.5–5.5 hr after infection as described (7). Cell lysates were prepared as described (7) except they were not precleared with antivirion serum, and 8×10^7 cells were lysed with 9 ml of phospholysis buffer (1% Triton X-100/0.5% sodium deoxycholate/0.1% $NaDodSO_4$/5 mM EDTA/0.1% aprotinin in phosphate-buffered saline). ^{32}P-Labeled virion RNA was isolated as described (12) from virions collected by centrifugation during preparation of the cell lysates. Synthetic VPg and coupled VPg-bovine serum albumin were gifts from Margaret Baron (7). ^{125}I-Labeled VPg was prepared by iodinating synthetic VPg on tyrosine with $Na^{125}I$ and Iodo-Beads (Pierce) as recommended by the manufacturer.

Anti-VPg serum and nonimmune serum from rabbits were prepared as described (7). Serum was passed through a column containing DEAE-Affi-Gel Blue overlaid with CM-Affi-Gel Blue as recommended by the manufacturer (Bio-Rad). The flow-through was brought to 45% $(NH_4)_2SO_4$ to precipitate IgG. The pellet was resuspended and dialyzed into 20 mM Hepes, pH 8.0/20 mM KCl/0.1 mM EDTA. This procedure removed protease and RNase activity.

Immunoprecipitations were done by incubating 200 μl of lysate supplemented with ovalbumin (0.5 mg/ml) and 36 milliunits ($A_{280} \times 10^{-3}$ unit) of purified IgG for 1 hr at 0°C. From a 10% suspension of *Staphylococcus aureus* cells, prepared by the method of Kessler (13), 50 μl was added and incubated for 30 min at 0°C. The cells were then washed once with Tris·HCl, pH 7.5/500 mM NaCl/1% Nonidet P-40/5% sucrose (vol/vol), then twice with H_2O. Cells were then boiled in H_2O for 3 min. This step did not detectably elute any synthetic VPg or VPg-pUpU from the bacterial cells but did significantly decrease the amount of nonspecifically bound material. About half of immunoprecipitated pre-VPg3 was lost by this treatment. Immunocomplexes were eluted from the cells by boiling for 3 min in 20 mM Tris·HCl, pH 7.5/10 mM dithiothreitol/2% $NaDodSO_4$/1 mM EDTA. Protein was precipitated with 10 vol of ethanol, solubilized by boiling for 3 min in 10 μl of sample electrophoresis buffer (10 mM sodium phosphate, pH 7.2/6 M urea/1% $NaDodSO_4$/1% 2-mercaptoethanol/0.02% bromophenol blue dye), and subjected to electrophoresis through a 12.5% polyacrylamide gel containing urea, $NaDodSO_4$, and sodium phosphate as described by Swank and Munkres (14). Gels containing [^{3}H]leucine were fluorographed with Enlightening (New England Nuclear) by soaking for 15 min without fixing; they were then dried and autoradiographed. Gels containing ^{32}P were dried without fixing and then autoradiographed. VPg and VPg-pUpU were lost from the gel if it was fixed (soaked in 10% acetic acid/30% isopropanol) and then fluorographed with EN^{3}HANCE (New England Nuclear). Protein gel markers from Bethesda Research Laboratories were prestained.

RNase T2 digestion was performed in 10 μl of 10 mM ammonium acetate (pH 4.5) with 2 units of enzyme and 1 μg of tRNA at 37°C for 1 hr. Digestion with nuclease P1 was at 37°C for 30 min in 10 μl of 10 mM ammonium acetate (pH 5.0), 1 μg of enzyme, and 1 μg of tRNA. Venom phosphodiesterase digestion was at 37°C for 60 min in 10 μl of 20 mM ammonium acetate, pH 9.0/5 mM $MgCl_2$/1 mM sodium phosphate/0.5 μg of enzyme (Worthington). Acid hydrolysis was carried out

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with 5.6 M HCl (freshly distilled) at 110°C for 1 hr and followed by repeated lyophilization. Nucleotides and peptides were separated by electrophoresis on cellulose thin-layer plates in pyridine/acetic acid, 0.5%:5% (pH 3.5) or in formic acid/acetic acid, 2.2%:7.8% (pH 1.9). VPg-pUpU was purified from immunoprecipitates by suspending the ethanol-precipitated protein in H_2O and passing it through a DEAE-Sephacel column (Pharmacia) equilibrated with H_2O. The flow-through was collected and lyophilized. VPg-pU was purified from nuclease P1 digestion products by binding the material to phosphocellulose in 25 mM ammonium acetate (pH 8.5), washing with the same buffer, then eluting with 250 mM ammonium acetate (pH 8.5) followed by lyophilization. VPg-pUpUpAp and VPg-pUp were prepared from virion RNA by RNase U2 and T2 digestion, respectively and immunoprecipitated with anti-VPg antibody as described above. RNase U2 digestion was in 15 μl of 20 mM Na citrate, pH 3.5/2 mM EDTA with 10 milliunits of enzyme and 10 μg of tRNA at 30°C for 30 min.

RESULTS

To determine whether free VPg is present in poliovirus-infected cells, proteins were immunoprecipitated with anti-VPg antibody from cytoplasmic extracts of infected HeLa cells labeled with [^{3}H]leucine during infection. Four viral proteins could be detected with anti-VPg antibody (Fig. 1A, lane 5). These four proteins were not precipitated with control antibody (lane 3) and their precipitation was blocked by synthetic VPg (lane 4). No cellular proteins, labeled with [^{3}H]leucine during a mock-infection, were recognized by control (lane 1) or anti-VPg (lane 2) antibody.

The four immunoprecipitated proteins were readily identified. The doublet at about M_r 13,000 corresponds in molecular weight to protein pre-VPg3, which has been identified previously as containing VPg determinants (7, 8). We do not know the reason for the double band. The protein just less than M_r 3,000 comigrates with iodinated synthetic VPg (lane M) and thus appears to be unmodified VPg. The third band is barely visible at M_r 4,200 and is VPg-pUpU, as will be shown below.

When the immunoprecipitations were repeated with lysates from cells labeled with $^{32}P_i$, a M_r 4,200 protein was evident after precipitation with anti-VPg antibody (Fig. 1B, lane 5) but not with control antibody or anti-VPg plus synthetic VPg (lanes 3 and 4). Again, no cellular proteins were immunoprecipitated (lanes 1 and 2). The ^{32}P-labeled protein was purified from immunoprecipitates by passage through a DEAE column (lane 6) and was shown to comigrate with the [^{3}H]leucine-labeled protein of M_r 4,200 (Fig. 1A, lanes 5 and 6). To examine whether the ^{3}H- and ^{32}P-labeled proteins behaved identically, both were cleaved with nuclease P1. After digestion, the molecular weights of both species were decreased to the same extent (Fig. 2, lanes 6–9).

From its electrophoretic mobility, it seemed that the M_r 4,200 species was VPg attached to several nucleotides. To examine its size more closely, its mobility relative to known markers was determined. The species (Fig. 2, lane 3) migrated between markers of VPg-pUpUpUAp (lane 2) and VPg-pUp (lane 4), the markers being derived from virion RNA. As another test, RNase T2-digested material was found to comigrate with the VPg-pUp marker (lane 5). These results suggest that the original molecule consisted of VPg attached to a dinucleotide.

To determine directly the structure of the M_r 4,200 material, it was analyzed by digestion with various nucleases followed by thin-layer electrophoresis. After purification, the material migrated as a single spot (Fig. 3A, lane 1). When the material was digested with snake venom phosphodiesterase, all of the radiolabel was removed as UMP (Fig. 4). Some P_i was generated from the UMP by a small amount of contaminating phosphatase. Therefore, VPg must be attached to a UMP residue(s) via a phosphodiester bond(s).

In other experiments, the purified protein was digested with nuclease P1 and about one-half of the radiolabel was released as UMP (Fig. 3A, lane 2). The protein now migrated faster toward the cathode than the undigested material. When nuclease P1-digested protein was purified (lane 3) and treated with snake venom phosphodiesterase, as expected, UMP was the only ra-

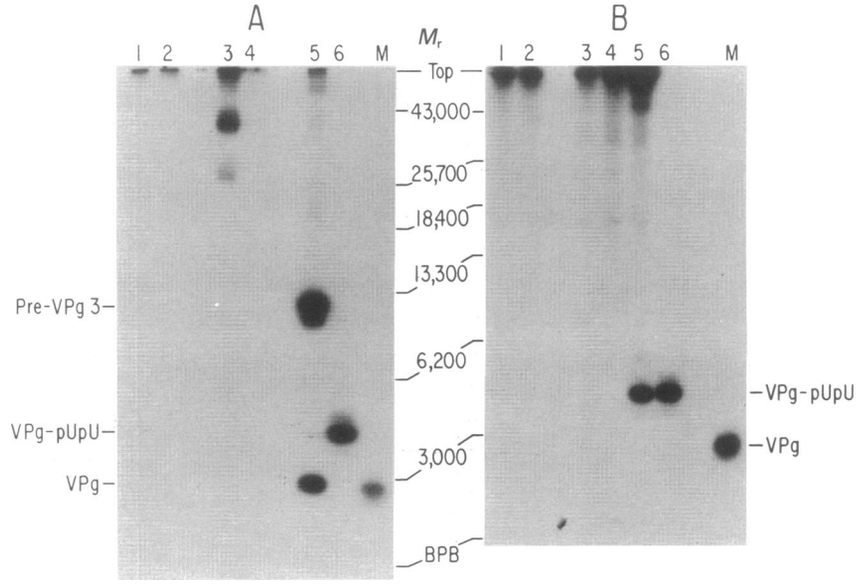

FIG. 1. Immunoprecipitation of VPg-related polypeptides by antiserum specific for VPg. (A) Cytoplasmic lysates were prepared from cells labeled with [^{3}H]leucine. Lysates from mock-infected cells were incubated with nonimmune immunoglobulin (lane 1) or anti-VPg antibody (lane 2). Lysates from poliovirus-infected cells were incubated with nonimmune immunoglobulin (lane 3), anti-VPg plus 10 μg of synthetic VPg (lane 4), and anti-VPg antibody (lane 5). Immunoprecipitates were prepared and analyzed by polyacrylamide gel electrophoresis. Purified ^{32}P-labeled VPg-pUpU (lane 6) and ^{125}I-labeled VPg (lane M) were included as markers. Bands in lane 3 are nonspecifically bound capsid proteins. (B) Cytoplasmic lysates were prepared from cells labeled with $^{32}P_i$. Lane designations are as described for A. BPB is bromphenol blue.

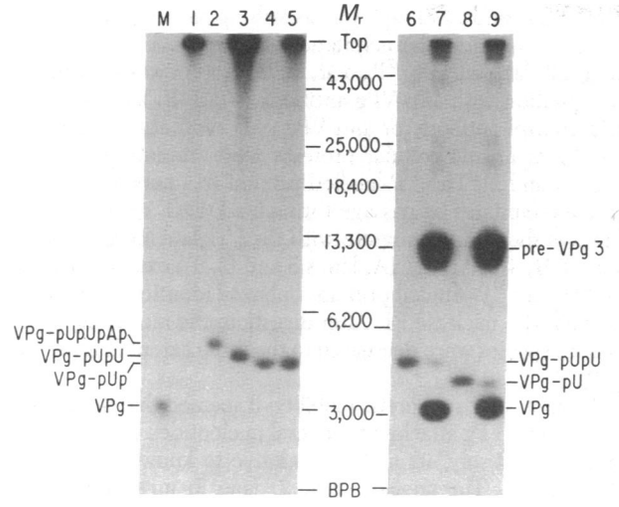

FIG. 2. Nuclease digestion of immunoprecipitated products. Lysates were prepared from poliovirus-infected cells labeled with $^{32}P_i$ or [^{3}H]leucine and incubated with antibody preparations. Immunoprecipitates were prepared and analyzed by polyacrylamide gel electrophoresis before or after nuclease digestion as follows: lane M, ^{125}I-labeled VPg marker; lane 1, ^{32}P-labeled immunoprecipitate, nonimmune immunoglobulin; lane 2, ^{32}P-labeled VPg-pUpUpAp marker prepared from virion RNA; lane 3, ^{32}P-labeled immunoprecipitate, anti-VPg antibody; lane 4, ^{32}P-labeled VPg-pUp marker from virion RNA; lane 5, ^{32}P-labeled immunoprecipitate, anti-VPg antibody, digested with RNase T2; lane 6, ^{32}P-labeled VPg-pUpU purified from anti-VPg immunoprecipitate; lane 7, [^{3}H]leucine immunoprecipitate, anti-VPg antibody; lane 8, nuclease P1 digestion products of purified ^{32}P-labeled VPg-pUpU; lane 9, [^{3}H]leucine immunoprecipitate, anti-VPg antibody, digested with nuclease P1. The gel containing [^{3}H]leucine (lanes 6–9) was prepared for fluorography with Enlightening, and both gels were then dried and autoradiographed. BPB is bromphenol blue.

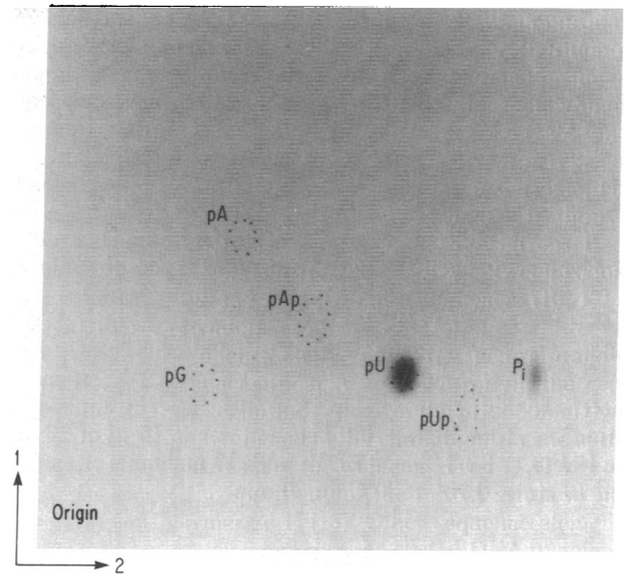

FIG. 4. UMP is the radiolabeled material attached to VPg. ^{32}P-Labeled VPg-pUpU was purified from anti-VPg immunoprecipitates. After digestion with snake venom phosphodiesterase, the material was spotted onto a cellulose thin-layer plate and chromatographed in two dimensions (first dimension, isobutyric acid/0.5 M NH_4OH, 5:3; second dimension, isopropanol/HCl/H_2O, 70:15:15). Nucleotide markers were visualized with UV light, and then the plate was autoradiographed.

diolabeled product (lane 4). Digestion of the purified protein (Fig. 3*B*, lane 1) with RNase T2 released no radiolabeled nucleotides (lane 2) but did remove a uridine residue from the protein because subsequent venom phosphodiesterase digestion released pUp (lane 4). In contrast, only UMP was generated by venom phosphodiesterase digestion of the original undigested material (lane 3). Therefore, the dinucleotide pUpU is attached to the protein via the 5′-phosphate of the first uridylate. If there had been a 3′-phosphate or more than two UMP residues in the chain, RNase T2 digestion would have released radiolabeled nucleotide(s), but it did not (Fig. 3*B*, lane 2). As will be shown later, the nucleic acid is attached to the amino

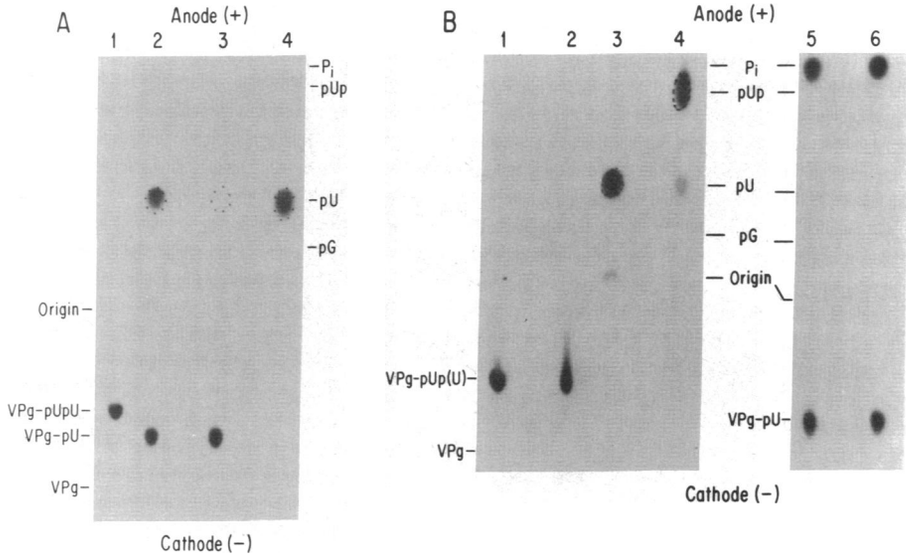

FIG. 3. Further characterization of ^{32}P-labeled VPg-pUpU. (*A*) Lanes: 1, ^{32}P-labeled VPg-pUpU purified from anti-VPg immunoprecipitates; 2, ^{32}P-labeled VPg-pUpU digested with nuclease P1; 3, VPg-containing nuclease P1 digestion product of ^{32}P-labeled VPg-pUpU, purified by phosphocellulose chromatography; 4, material from lane 3 digested with snake venom phosphodiesterase. Material was spotted onto a cellulose thin-layer plate, subjected to electrophoresis at pH 3.5, and visualized by autoradiography. (*B*) Lanes: 1, ^{32}P-labeled VPg-pUpU; 2, ^{32}P-labeled VPg-pUpU digested with RNase T2; 3, ^{32}P-labeled VPg-pUpU digested with snake venom phosphodiesterase; 4, ^{32}P-labeled VPg-pUpU digested with RNase T2 and then with snake venom phosphodiesterase; 5, ^{32}P-labeled VPg-pUpU digested with RNase T2 and then with nuclease P1; 6, ^{32}P-labeled VPg-pUp obtained from virion RNA (see Fig. 2, lane 4) digested with nuclease P1 and used as a marker. Material was analyzed as in *A*.

acid tyrosine in VPg. Because VPg contains only one tyrosine residue, we conclude that the M_r 4,200 protein is modified with only one UMP dinucleotide.

These data also strongly indicate that the protein moiety in the ^{32}P-labeled species is VPg. It is a polypeptide specifically recognized by anti-VPg antibody (Fig. 1) and present in virus-infected but not in mock-infected cells (Fig. 1). The RNase T2-digested material (VPg-pUp) has the same apparent molecular weight as VPg-pUp from virion RNA (Fig. 2, lanes 4 and 5). In addition, nuclease P1-digested material (VPg-pU) comigrated with VPg-pU from virion RNA (Fig. 3*B*, lanes 5 and 6) during thin-layer electrophoresis.

Finally, the linkage between VPg and the UMP dinucleotide was determined. Because the linkage between VPg and virion RNA is known (O^4-phosphotyrosine), VPg-pU was isolated from virion RNA and used as a standard. ^{32}P-Labeled VPg-pU prepared from immunoprecipitates (Fig. 3*A*, lane 3) and from virion RNA was hydrolyzed with HCl. The hydrolysates were analyzed by electrophoresis on cellulose thin-layer plates at pH 1.9 (Fig. 5*A*) and at pH 3.5 (Fig. 5*B*). All spots present in the hydrolysate of VPg-pU from immunoprecipitates (Fig. 5, lanes 2 and 4) were also present in the standard (lanes 1 and 3). Two spots were not immediately identified by internal markers. One spot (unmarked) is probably a partial degradation product. The other (marked Tyr-pU) was eluted and shown to contain only O^4-phosphotyrosine by subsequent digestion with micrococcal nuclease or with periodate treatment and base-catalyzed β-elimination (data not shown). Therefore, as in virion RNA, the linkage between VPg and the UMP dinucleotide is O^4-phosphotyrosine. The structure of the M_r 4,200 protein is thus VPg(Tyr-O^4)-pUpU.

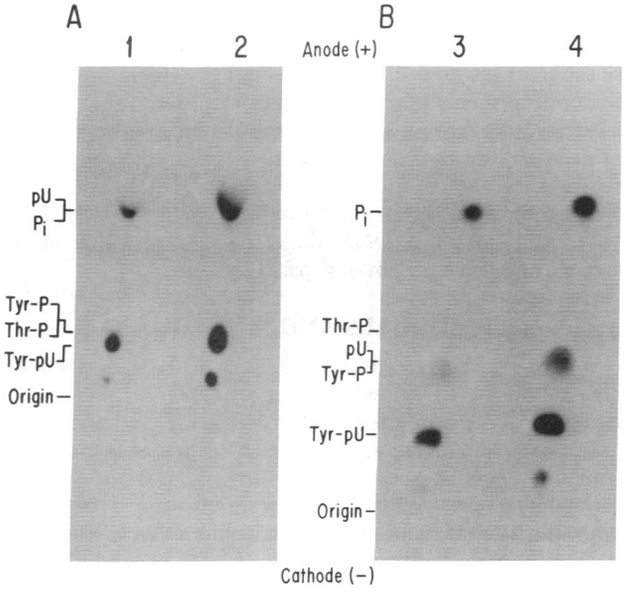

FIG. 5. UMP dinucleotide is linked to tyrosine in VPg-pUpU. (*A*) Lanes: 1, ^{32}P-labeled VPg-pU prepared from virion RNA (see Fig. 3*A*, lane 3); 2, ^{32}P-labeled VPg-pU from immunoprecipitates of protein from infected cells. Protein was hydrolyzed with HCl and then lyophilized. Material was spotted onto a cellulose thin-layer plate and subjected to electrophoresis at pH 1.9 for 5 min (to the right in the figure) then for 25 min (upward in the figure). The thin-layer plate was then autoradiographed. UMP marker was visualized with UV light; phosphotyrosine and phosphothreonine were visualized with ninhydrin. (*B*) Same as *A* except material from virion RNA (lane 3) and immunoprecipitates (lane 4) was subjected to electrophoresis at pH 3.5.

DISCUSSION

We have detected free cytoplasmic VPg and VPg-pUpU in poliovirus-infected cells. Previous attempts to find free VPg (7, 8) probably failed because VPg leached from the polyacrylamide gels during fluorography or fixation. We minimized this problem by omitting the fixation step and by rapid fluorography of gels containing [^{3}H]leucine-labeled material using Enlightening.

There are several possible origins for VPg and VPg-pUpU. They could be synthesized in the cell by direct enzymatic processing of precursor polypeptides. Alternatively, VPg could be released from plus strand RNA when it is processed into mRNA by the unlinking enzyme that cleaves the O^4-phosphotyrosine bond between VPg and RNA (15). VPg-pUpU could be a RNA degradation product or it could be a premature termination product of RNA replication that accumulates late in infection.

Our discovery of free VPg and VPg-pUpU in poliovirus-infected cells increases the number of possible primers for poliovirus RNA replication. In addition to the previously described precursors of VPg (7–9), VPg or VPg-pUpU could initiate RNA synthesis. The structure of the 5′ end of plus and minus strand RNA is VPg-pUpU so that if free VPg-pUpU were an initiator, the correct 5′ end would be generated without the need for further processing. A very attractive model for initiation would be synthesis of VPg-pUpU without a template, its hybridization to template RNA, and elongation by replicase.

In parallel work, Takegami *et al.* (16) have found that VPg-pUpU is made *in vitro* in membrane preparations from poliovirus-infected cells.

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